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SHORT COMMUNICATION

Molecular cytogenetic studies in western Mediterranean *Juniperus* (Cupressaceae): a constant model of GC-rich chromosomal regions and rDNA loci with evidences for paleopolyploidy

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Abstract We carried out the first cytogenetic characterisation of Mediterranean species of *Juniperus* (Cupressaceae); to date, nuclear DNA amount and chromosome numbers were known, but a fine karyotype analysis had been only done in three eastern Asian taxa. We performed chromomycin A_3 banding, for the first time in the family, and fluorescent *in situ* hybridisation (FISH) in order to detect 18S-5.8S-26S (also termed 35S and 45S) rRNA genes in five species of the genus, four diploid (*J. communis*, *J. oxycedrus*, *J. phoenicea* and *J. sabina*) and the only Mediterranean one that is exclusively tetraploid (*J. thurifera*). The results show a very homogeneous pattern, with one chromosome pair with chromomycin-positive bands at the secondary constrictions and colocalised 35S rDNA signals. In diploid species, this model agrees with that of the two Asian taxa previously investigated with FISH. In the tetraploid species, conversely, the results are different: in the Asian taxa signal number and location are exactly double in tetraploid than in diploid taxa, whereas in our case the tetraploid species bears the same number of signals (not twice) as diploid ones. This fact can be interpreted as accounting for the age of polyploidy in *J. thurifera*: after the polyploidisation event and the stabilisation of the doubled genome, there has been time left for genome rearrangements implying a loss of GC-rich chromatin and one of the rDNA loci; this argument is a support for the ancient and relict character attributed to this taxon.

Keywords FISH, fluorochrome banding, gymnosperms, *Juniperus*, karyotype, Mediterranean area, polyploidy.

Introduction

With around 75 species and 100 taxa including infraspecific levels (Adams and Schwarzbach 2013), the genus *Juniperus* L. is the largest one of its family (Cupressaceae) and the second in the gymnosperms after *Pinus* L. It is very largely and abundantly distributed in Northern hemisphere, from sea level to mountains and from subtropical to arid zones, whereas it is extremely rare in the Southern hemisphere, with only one African taxon (Nagano et al. 2007; Mao et al. 2010; Adams and Schwarzbach 2013; Romo et al. 2013). With two conspicuously distinct morphological patterns, acicular- and squamiform-leaved, the genus is monophyletic and is divided in three at their turn monophyletic sections: *Caryocedrus* Endl., comprising only one eastern Mediterranean species (*J. drupacea* Labill.), *Juniperus*, with around 14 mostly Mediterranean taxa (including *J. communis* L., also present in the Mediterranean, but subcosmopolitan), and *Sabina* Spach, including the remaining taxa, distributed in all Northern Hemisphere continents plus southern Africa (Adams and Schwarzbach 2013, and references therein). The Mediterranean region is one of the relevant diversity spots of the genus, hosting the three sections and 15 taxa being endemic of its western part (Gauquelin 2006; Mao et al. 2010). A high number of *Juniperus* taxa have economic value in medicine, food and other industries (Romo et al. 2013, and references therein).

Data regarding chromosomes (number, karyotype morphometry, physical mapping of several regions and/or genes) together with information on nuclear DNA amount, are fundamental for establishing the genome organisation and its variation across the plant groups, which constitutes a relevant tool for biogeographic, systematic, phylogenetic and evolutionary studies (Bennett 1998; Levin 2002). With chromosome number available from around one third of the species of the genus (Index to Plant Chromosome Numbers, IPCN,

http://www.tropicos.org/projectwebportal.aspx?pagename=Home&projectid=9, accessed August 22, 2014), *Juniperus* has only one base number, *x*=11, almost all taxa being diploid (2*n*=22), consistently with the rarity of polyploidy in gymnosperms (5%; Ahuja 2005, and references therein). Nevertheless, a few triploid and tetraploid taxa (or in some cases just cytotypes, not present in all the populations) have been reported in *J. chinensis* L. (two cultivars, one subspecies and one variety; Sax and Sax, 1933; Nagano et al. 2000, 2007; Zonneveld 2012), *J.* x*pfitzeriana* (Spath) Schmidt (two cultivars; Zonneveld 2012), *J. sabina* L. (some populations and one cultivar; Jensen and Levan 1941; Muratović et al. 2004; Siljak-Yakovlev et al. 2010; Zonneveld 2012), *J. squamata* Buch.-Ham. (one variety; Jensen and Levan 1941) and *J. virginiana* L. For *J. gamboana* Martínez and *J. wallichiana* Hook.f. & Thomson ex Parl. the tetraploid level is suggested by only one chromosome count in each case (IPCN, see address and access date above). A population of *J. foetidissima* Willd. has been detected with a nuclear DNA amount suggesting a hexaploid level (M. Bou Dagher-Kharrat and S. Siljak-Yakovlev, unpubl. res.). To date, the only species having been found exclusively polyploid (tetraploid, 2*n*=44), with many populations studied all along its distribution area, is *J. thurifera* L. (Horjales et al. 2003; Romo et al. 2013). Genome size, indicative of ploidy levels, has been assessed in around one quarter of *Juniperus* species (Horjales et al. 2003; Loureiro et al. 2007; Murray et al. 2012; Romo et al. 2013). In contrast to this relatively high degree of karyological and cytogenetic knowledge, molecular cytogenetic information is very scarce in the genus. No works have been done on chromosome banding, and fluorescent *in situ* hybridisation (FISH) has only been performed on three taxa (*J. chinensis* var. *procumbens* Endl., *J. chinensis* var. *sargentii* A.Henry and *J. lutchuensis* Koldz.; Nagano et al. 2007); additionally, *J. californica*

Carr. has been investigated for rDNA structure, though not physically mapping on chromosomes (Garcia and Kovařík 2013).

The objective of this paper is to contribute to the detailed karyotype characterisation by investigating GC-rich regions and 35S rDNA loci distribution for the first time in Mediterranean species of the genus. We aim at completing data to the karyological and cytogenetic knowledge of the taxa already studied from chromosome number and genome size viewpoints in Romo et al. (2013) in order to shed light on their genome evolution and relationships.

Materials and methods

Plant material

We selected five taxa reported in Table 1 of Romo et al. (2013), all of them coming from wild populations in Spain: the four corresponding to the typical infraspecific taxa (variety or subspecies: *J. communis* L. var. *communis*, *J. oxycedrus* L. subsp. *oxycedrus*, *J. phoenicea* L. subsp. *phoenicea* and *J. thurifera* subsp. *thurifera*) plus the species without taxonomically described infraspecific diversity (*J. sabina*). One population per taxon was studied and in all cases root tips were collected from plants cultivated in pots in the Jardí Botànic de Barcelona. This choice is comprehensive of the genus diversity at morphological (taxa with acicular and with squamiform leaves), taxonomic (members of the two main sections, the third one being monotypic) and cytogenetic (representatives of diploid and tetraploid cytotypes) levels.

Protoplast preparation and idiogram elaboration

Root tips were pretreated with 0.05% aqueous colchicine for 16 h at room temperature and fixed in absolute ethanol and glacial acetic acid (3:1) at room temperature for 1-2 h, then 2-4 days at 4ºC and finally transferred to 70% ethanol and kept at 4ºC. Root tips were washed for 10 min in pH=4.6 0.01 M citrate buffer with shaking. The meristematic part of root tips was digested on an enzyme mix [4% RS cellulase (Yakult Honsha Co. Tokyo, Japan), 1% pectolyase Y-23 (Seishin Co. Tokyo, Japan), 4% hemicellulase (Sigma)] at 37ºC during 5 h, then transferred to distilled water at room temperature for at least one hour. Meristems were squashed onto a drop of freshly prepared 45% acetic acid and the preparations were observed at a phase contrast microscope. The best slides were frozen at -80 ºC overnight and then the coverslips were removed and the slides were rinsed with absolute ethanol and air-dried.

Each idiogram was elaborated based on five metaphase plates stained with 4′,6-diamidino-2-phenylindole (DAPI), obtained in the FISH experiments (see next subheading). Chromosome arms were measured both manually and with the help of the MicroMeasure v. 3.3 (Colorado State University, USA).

Fluorochrome banding and FISH

Chromomycin A_3 (CMA, Serva) banding was carried out for the detection of GC-rich regions, following the technique of Schweizer (1976) with minor modifications from Siljak-Yakovlev et al. (2002) concerning the concentration of chromomycin (0.2 mg/ml) and time of staining (60 min).

FISH was performed for the detection of 35S rDNA loci. The 35S rDNA probe was a 4 kb clone from the *Eco*RI fragment, including 18S-5.8S-26S rDNA sequences

from *Arabidopsis thaliana* (L.) Heynh., labelled with the direct Cy3 fluorochrome (Amersham, Courtaboeuf, France) by nick translation, according to the manufacturer's protocol. For *in situ* hybridisation the method of Heslop-Harrison et al. (1991) was used with the following modifications for gymnosperms from Siljak-Yakovlev et al. (2002) and Bogunić et al. (2011): the treatments with RNase and pepsin lasted respectively 60 and 20 min; the slides were denatured in 70% or 50% formamide (Sigma Aldrich Co., Steinhem, Germany) in 2xSSC for 2 min at 70 ºC, then dehydrated in an ethanol series for 3 min each (70%, 90% and 100%) and dried; the hybridisation was performed for 36 h at 37 ºC. Slides were counterstained and mounted in Vectashield medium containing DAPI (Vector Laboratories, Burlingame, CA, USA).

Preparations were observed, using the adequate combinations of excitation and emission filters (01, 07, 15 and triple filter set 25), on a Zeiss Axiophot epifluorescence microscope, where the best metaphase plates were photographed with a CCD camera (RETIGA 2000R; Princeton Instruments, Evry, France) and an image analyse program (Metavue, Evry, France). Several good quality metaphase plates from different root tips were analysed for each taxon.

Results and discussion

Chromosome numbers and data on GC-rich regions and 35S rDNA loci of the taxa studied are presented in Table 1. Metaphase plates of the five species with banding and FISH signals appear in Fig. 1 (a-e). Given the constancy of both karyotype morphometric model and banding and FISH patterns, haploid idiograms with the location of chromomycin-positive (CMA+) bands and rRNA genes are provided for three taxa, representative of the two sections and two ploidy levels (Fig. 1, f-h). Table 2

summarises the 35S rDNA FISH data available for the gymnosperm genera in order to have a comparative idea of their variability.

Chromosome number and karyotype morphometry

Chromosome numbers and ploidy levels of the taxa considered, already studied in Romo et al. (2013), have been confirmed in all cases. The numbers ($2n=2x=22$ and $2n=4x=44$) agree with those found in other species and support $x=11$ as the basic chromosome number of the genus (IPCN, see address and access date in the introduction).

Idiograms, indicating karyotype morphometric details, are provided for the first time for the taxa studied. Previously, Nagano et al. (2000) also built idiograms for several eastern Asian representatives of the genus. Our data show a symmetric pattern, both for inter- and intrachromosomal symmetry, with metacentric and submetacentric similar-sized chromosomes, including one pair bearing a secondary constriction (SC). This model is basically coincidental with that reported for the above-mentioned Asian taxa, the most important difference being that the tetraploid species here considered (*J. thurifera*) has only one chromosome pair with SC instead of two as it is the case for several tetraploid *J. chinensis* varieties (Nagano et al. 2000). Regarding symmetry, the present results agree not only with the aforementioned previous ones on *Juniperus*, but with the general pattern in gymnosperms, which exhibit symmetrical karyotypes with mostly metacentric and some submetacentric chromosomes (Bogunić et al. 2011, and references therein).

Chromosomal GC-rich regions

CMA+ bands (GC-rich regions) have been detected for the first time in *Juniperus* and in the family Cupressaceae. They are in number of two in all studied taxa (both diploid and tetraploid one) and appear in the SC- bearing chromosomal pair. This pattern differs from those described in other gymnosperm genera both by the constancy and the scarcity of CMA+ bands. From four to 26 bands have been reported in several genera, such as *Abies*, *Cedrus*, *Larix*, *Picea* and *Pinus*, the number varying among taxa (Hizume et al. 1994; Bou Dagher-Kharrat et al. 2001; Siljak-Yakovlev et al. 2002; Puizina et al. 2008; Bogunić et al. 2011). GC-rich regions have also been reported in SC, though not only there, in *Picea* (Siljak-Yakovlev et al. 2002).

35S rDNA loci

Data on rRNA gene location are provided here for the first time for all the studied taxa. As reported for fluorochrome banding, rDNA FISH pattern is also highly constant.

Two 35S rDNA signals appear in both sides of the SC in all studied species. These signals are colocalised with CMA+ bands, indicating that 35S rRNA genes are located in GC-rich regions, as stated for many other gymnosperm genera (Bogunić et al. 2011, and references therein). This rDNA model almost absolutely agrees with the one of previously studied Asian *Juniperus* taxa (Nagano et al. 2007). The number and location of 35S rDNA loci are the same in the diploid taxa, whereas in the tetraploid species here considered there is not a loci duplication (as it occurs in the Asian one), but only one locus (two signals) is observed, in agreement with the above-mentioned fact that there is only one chromosome pair with SC in *J. thurifera*. This 35S rDNA pattern is the simpliest in gymnosperms, where from two (one locus) to 26 signals (13 loci)

have been reported (see Table 2 and references there provided). Two signals are only found, apart from *Juniperus*, in another genus belonging to the family Cupressaceae (*Cunninghamia*), and in *Larix* (Pinaceae), *Microcycas* (Cycadaceae) and *Podocarpus* (Podocarpaceae). The other representative of the Cupressaceae studied (*Cryptomeria*) presents four bands (two loci), suggesting that this family is characterised, within the gymnosperms, by a low number of rDNA loci. The location in a SC agrees with that of the other *Juniperus* taxa investigated (Nagano et al. 2007), and other studies on gymnosperms (Table 2) mention this structure too, although different positions are also reported.

Genome organisation in *Juniperus* and evidences for paleopolyploidy

The present results obtained on *Juniperus* allow us to depict a rather simple genome organisation in terms of karyotypic data: a very symmetrical karyotype with a low and very constant and conserved fluorochrome banding and rDNA FISH pattern. Although karyotype symmetry is common to all gymnosperms, this very low degree of variation in GC-rich regions and rDNA sites seems characteristic of the genus *Juniperus* and the family Cupressaceae. No distinction can be done on the basis of karyotype features between the two biggest sections of the genus, *Juniperus* and *Sabina*, which are, conversely, very well characterised by a morphological trait (acicular and scale-shaped leaves, respectively), and which agree with the molecular phylogenetic structuring of the genus (Adams and Schwarzbach 2013). Rai et al. (2008) showed *Juniperus* (and, in general the family Cupressaceae) in one of the most derived clades in the conifers. This leads to consider a tendency towards a simplification in karyotype organisation along the evolutionary pathway in this group.

One karyological trait is significantly distinctive in *Juniperus* among gymnosperms: polyploidy has been detected in eight species (see introduction), meaning around 10% of the genus, which is double than the polyploidy presence in the gymnosperms (around 5%; Ahuja 2005). This indicates that genome multiplication (to date to triploid and tetraploid levels), a very typical evolutionary mechanism in plants (Cui et al. 2006, and references therein), plays in *Juniperus* a bigger role than in gymnosperms in general.

Within this karyotypic homogeneity, the fact that only one chromosome pair bears a SC, where GC-rich regions and 35S rRNA genes are located, in the tetraploid *J. thurifera* is highly interesting. As stated above, this Mediterranean taxon does not show the *a priori* expectable double number of CMA+ bands and 35S rDNA signals in respect of related diploids, as it is the case in the eastern Asian *J. chinensis* var. *procumbens* (Nagano et al. 2007). This fact can be interpreted as accounting for the age of polyploidy in *J. thurifera*: after the polyploidisation event and the stabilisation of the doubled genome, there has been time left for genome rearrangements implying a loss of GC-rich chromatin and one of the rDNA loci, whereas this has not occurred (almost not yet) in the Asian taxon, most probably younger. The different taxonomic treatments of the two entities (one variety in the Asian taxon and one species, split in two subspecies -both tetraploid-, in the Mediterranean one) also claim for a bigger differentiation, within the speciation process, of the taxon that we believe to be more ancient. *Juniperus thurifera* is considered an old species, originated in the Tertiary, which, particularly in the cold periods of the Pleistocene, had reached a bigger extension than it has now, and especially a denser distribution with a larger number of populations, their current ones being qualified of relict in a fragmented area (Gauquelin et al. 1999; Montesinos 2007; Terrab et al. 2008; Villar 2013). The present cytogenetic data provide a fully concordant

argument with the ancient and relict character attributed to this species, which may be qualified as paleopolyploid and fits in the concept of paleoendemic as recently redefined by Siljak-Yakovlev and Peruzzi (2012).

Concluding remarks and perspectives

This is the first detailed karyotype analysis in five Mediterranean representatives of *Juniperus*. Karyology and molecular cytogenetics show a very constant and simple genome organisation at the chromosomal level, basically coincidental with that of the previously studied three Asian taxa of the genus. *Juniperus* presents a very characteristic karyotypic pattern, which does not permit any infrageneric differentiation. Polyploidy plays a bigger role in *Juniperus* than in gymnosperms in general. Within polyploids, *J. thurifera* presents the only differential karyotypic character, not doubling the GC-rich regions and the rDNA sites. Further cytogenetic studied in the genus should be mostly centred on taxa in which polyploidy has been detected (such as *J. foetidissima* and *J. sabina* regarding Mediterranean species) in order to better assess the role of this mechanism in the evolution of the genus and the relationships between karyological and cytogenetic data on the one hand and differentiation, speciation and evolutionary rate on the other side.

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Data archiving statement. FISH data are available at Plant rDNA database (http://www.plantrdnadatabase.com).

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Figure caption

Fig. 1 Metaphase plates of the studied taxa showing 35S rDNA loci (a-d) and chromomycin-positive bands (e), and idiograms of three taxa, representing both ploidy levels (diploid and tetraploid) and both sections (*Juniperus*, *Sabina*) studied, with the GC-rich regions bands (yellow) and 35S rDNA loci signals (red). a: *Juniperus oxycedrus*; b: *J. communis*; c: *J. phoenicea*; d, e: *J. thurifera*; f: *J. oxycedrus* (2*x*, sect. *Juniperus*); g: *J. phoenicea* (2*x*, sect. *Sabina*): h, *J. thurifera* (4*x*, sect. *Sabina*). Scale bars = 5 µm.